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Cadherin-11 is unique amongst cadherins in that it is exists as two alternatively spliced forms that are expressed together in the same cell. In year one of this grant we showed that the presence of the cadherin-11 splice variant promotes invasion of cadherin-11 positive breast cancer cells, perhaps by promoting cell-ECM interactions. In year two of the grant we showed that inhibition of cadherin-11 function using proprietary small molecule cadherin-11 inhibitors influences the ability of cadherin-11-expressing cells to invade in vitro. In year three of the grant we continued our work on the small molecule inhibitors of cadherin-11 and characterized cells lines expressing cadherin-11 siRNA. Our work also shows effects of cadherin-11 blockade on the expression of the angiogeneic molecules VEGF-A and VEGF-D. In other studies we investigated the regulation of cadherin-11expression in breast cancer cells and showed marked effects of cell density and of wnt and TGF-beta signaling.

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Introduction:

Cadherins play a critical role in establishing adherens-type junctions by mediating Ca^{2+}-dependent cell-cell adhesion. Specific homophilic cell-cell adhesion plays a key role in tissue and organ development during embryogenesis and in maintenance of normal tissue structure in adult organisms (Ivanov *et al.*, 2001). Cadherin-based cell-cell adhesion is involved in early embryonic morphogenesis. For example, mice lacking E-cadherin exhibited early embryonic lethality (Riethmacher *et al.*, 1995)

Cadherins are transmembrane glycoproteins that participate in the formation of homophilic cell-cell adhesion complexes. An extracellular protein domain consisting of a varying number of highly homologous cadherin domains structurally characterizes cadherins. Classical cadherins contain five extracellular cadherin domains harboring calcium-binding sites that are located between neighboring extracellular repeats (Ivanov *et al.*, 2001). It was found that stability of the cadherin protein and subsequent cadherin-mediated cell-cell adhesion was dependent on Ca^{2+} binding to the extracellular domain of the protein.

The intracellular protein domain of cadherins serves to bind cytoplasmic proteins including $p120^{ctn}$, β-catenin and plakoglobin. Hereby, β-catenin acts as a linker between the cadherin domain and α-catenin. Further, the catenins mediate the interaction between the cadherin protein and the cytoskeleton including actin and vinculin (Yagi *et al.*, 2000).

Cadherin-11 was first cloned independently from a mouse osteoblastic cell line MC3T3-E1 and mesodermally derived tissues (Okazaki *et al.*, 1994; Hoffman *et al.*, 1995). During development cadherin-11 was expressed predominantly in mesenchymal components of organs. In contrast, E-cadherin was found exclusively in epithelial structures, as was N-cadherin. Cadherin-11 appeared to be down-regulated upon differentiation of tissues (Hoffman *et al.*, 1995).

A cadherin-11 isoform, termed cadherin-11 variant was found to encode a truncated protein with an altered cytoplasmic domain. The cytoplasmic protein domain resembled no part of the cytoplasmic domain in other cadherins. Gene analysis revealed an insertion of 179 bp in the transmembrane domain hence generating an alternatively spliced form (Kawaguchi *et al.*, 1999). The protein size of the cadherin-11 variant form is ~85 kd in comparison to full-length cadherin-11 with a size of ~120 kd. The cad-11 variant form is unable to associate with β-catenin as the cytoplasmic domain does not possess a catenin-binding site. Cell aggregation studies suggested the variant form to enhance adhesion kinetics when L cells were transfected with cadherin-11 or cadherin-11 in combination with the variant form. In addition, it was suggested that cadherin-11 variant enhances the association between cadherin-11 and the cytoskeleton via β-catenin thereby stabilizing the full-length cadherin-11 (Kawaguchi *et al.*, 1999). Distribution of variant mRNA in tissues and cells appeared the same as cadherin-11 but expression levels were lower.

Previously, loss of expression or function of the epithelial cell-cell adhesion molecule E-cadherin was found to be associated with a loss of epithelial phenotype and with a gain of invasiveness in a number of cancers including breast cancer (Pishvaian *et al.*, 1999). In contrast expression of cadherin-11 and its variant form were found to be expressed in the most invasive breast cancer cell lines but were not detected in non-invasive cell lines (Pishvaian *et al.*, 1999). Further, it was proposed that expression of the cadherin-11 splice variant promotes invasion of cadherin-11-positive breast cancer cells (Feltes *et al.*, 2002). Hence cadherin-11 expression was suggested to be correlated with the invasive phenotype in cancer cells and could potentially serve as a predictor of the invasive and metastatic phenotype.

The vascular endothelial growth factors have been implicated in the progress of angiogenesis *in vivo* and *in vitro* and lymphangiogenesis (Marconcini *et al.*, 1999). The family of vascular endothelial growth factors (VEGFs) encompasses six proteins including VEGF-A, -B, -C, -D, placenta growth factor (PlGF) and the viral homologues, collectively called VEGF-E (Karkkainen *et al.*, 2000). VEGFs all possess a VEGF homology domain that spans approximately 100 amino acids and is characterized by the precise spacing of 8 cysteine residues. VEGFs are specific ligands that bind to VEGF-receptors (VEGFR) in order to initiate molecular processes involved in angiogenesis and lymphangiogenesis. PlGF and VEGF-B bind to VEGFR-1 whereas VEGF-A interacts with both VEGFR-1 and VEGFR-2. VEGF-C and VEGF-D bind VEGFR-2 and VEGFR-3 (Achen *et al.*, 1998; Karkkainen *et al.*, 2000).

Expression studies of VEGF family members in breast cancer cell lines found all VEGFs to be expressed in the seven cell lines analyzed, despite at varying levels (Kurebayashi *et al.*, 1999). Further investigation into the expression levels of VEGFs in human breast tumors revealed VEGF-A and VEGF-B to be expressed in both node-positive and - negative tumors while VEGF-C expression was only detected in node-positive tumors. VEGF-D was detected in inflammatory breast cancer and a tumor that had developed an inflammatory skin metastasis (Kurebayashi *et al.*, 1999). An additional study concerned the prognostic value of the splice variants of VEGF-A with respect to expression levels in breast and ovarian cancer (Stimpfl *et al.*, 2002). Results showed $VEGF^{121}$ and $VEGF^{165}$ to be the most dominantly expressed variants of VEGF-A in all tumor samples and cell lines analyzed whereas $VEGF^{145}$ was weakly or not expressed in breast and ovarian cancers. No correlation between VEGF-A splice variant expression in tumors and histological type, differentiation grade, tumor size and nodal status from cancer patients was found. Therefore, the study concluded that no correlation between the invasive capacity of breast cancer cell lines and VEGF-A isoforms was apparent (Stimpfl *et al.*, 2002).

Finally, studies found expression of VEGF-D to be induced by cadherin-11 mediated cell-cell interaction in fibroblasts (Orlandini *et al.*, 2001). It was demonstrated that in non-interacting cell expression of VEGF-D was low while VEGF-D expression was high in contacting cells. Calcium deprivation and its associated loss of cadherin-11 from the cell surfaces also resulted in subsequent loss of VEGF-D transcript. Inhibition of cadherin-11 using specific anti-sense RNA constructs resulted in a loss of VEGF-D mRNA in confluent BALB/c fibroblasts. In contrast, increased expression of cadherin-11 in NIH3T3 cells led to induction of VEGF-D expression (Orlandini et al., 2001).

In conclusion, the above reports suggest that cadherin-11 might be able to facilitate breast cancer cell invasion resulting in angiogenesis and lymphangiogenesis and therefore examination of the mechanism by which cadherin-11 promotes invasion and the relationship of cadherin-11 to VEGF and cell invasion is essential.

Materials and methods:

Cell lines: All cell lines were obtained from American Type Culture Collection and grown in DMEM supplemented with 5% fetal bovine serum. Cells were incubated at 37°C with 5% CO_2.

RNAi: RNAi describes the mechanism of introducing small double-stranded RNA oligonucleotides into cells in order to generate RNA induced silencing complexes that subsequently degrade specifically targeted endogenous mRNA through endonuclease activity. For the production of double-stranded RNAi, appropriate templates were ordered from Integrated DNA Technologies, Inc.. All templates contained the T7 promoter sequence at the 3' end of the sequence as well as an AA 5' overhang (**table 1**). Double-stranded RNAi was synthesized using the Silencer™siRNA Construction Kit (Ambion).

Function	Template name	Template sequence (5' to 3')
anti-cadherin-11 WT	17 anti-sense	AAC AGC GTG GAT GTC GAT GAC CCT GTC TC
	17 sense	AAG TCA TCG ACA TCC ACG CTG CCT GTC TC
anti-cadherin-11 WT and cadherin-11 variant	55 anti-sense	AAC CCA CCA AAG TTT CGG CAG CCT GTC TC
	55 sense	AAC TGC CGA AAC TTT GGT GGG CCT GTC TC
anti-cadherin-11 variant	123 anti-sense	AAG CTT AAT GGA ACC CCC CTC CCT GTC TC
	123 sense	AAG AGG GGG GTT CCA TTA AGC CCT GTC TC

Table 1: Sequence of RNAi templates used in gene silencing experiments.

Transient transfection and gene silencing assays: Cells were grown to 30-50% confluency before being transfected with RNAi using oligofectamine according to the protocol supplied. Briefly, the RNAi oligonucleotide and oligofectamine were diluted in serum-free Opti-MEM® I in separate Eppendorf tubes before being combined following a 10-15 minutes incubation at RT. The combined solution was further incubated for 15-20 minutes at RT. The resulting RNAi complexes were added to the cells and incubated at 37°C with 5% CO_2 for 4 hours. Following incubation 15% FBS-containing DMEM was added. Cells were further analyzed after 72 hours or after 3 consecutive RNAi transfections where appropriate.

RNAi vector-based gene silencing assays: Cells were grown to 30-50% confluency before being transfected with RNAi vector constructs (Ambion) and hygromycin B resistance vectors using Fugene according to protocol supplied. Cell colonies were selected in hygromycin B containing growth medium. A number of colonies were pooled to produce pooled stable transfectant cell lines.

Immunoblotting: Cells were lysed in a standard SDS-sample buffer at room temperature (RT). Samples were spun at 14,000 rpm for 10 minutes to remove insoluble cell debris and boiled for 10 minutes. A Bio-Rad protein assay was performed to determine total protein content. After addition of 10% β-mercaptoethanol and bromo-phenolblue samples were boiled for a further 10 minutes. Equal total protein was loaded on 3-8% NuPage Tris-acetate gels. Proteins were blotted to nitrocellulose and blocked for 1 hour at room temperature in 5% milk-PBST. Following incubation with appropriate primary (at 4° overnight) and secondary (at RT for 1 hour) antibodies, blots were treated with enhanced chemiluminescence reagent and exposed to film.

Immunocytochemistry: Double immunofluorescent staining for β-catenin and cadherin-11 or E-cadherin was performed on cells plated out on coverslips in 12-well plates at equal numbers. Cells were washed 3 times in PBS and fixed in 2% paraformaldehyde in PBS for 30 minutes. Cells were then permeabilized in 0 .2% Triton-X-100 in PBS for 5 minutes at room temperature. Cells were blocked in 6% normal goat serum, in PBS, at 4°C overnight. 5B2H5 (cadherin-11) or E-cadherin antibody diluted 1:200 in normal goat serum was applied to and incubated on the cells at 4°C overnight. Cells were washed 3 times in PBS. The cells were incubated with SHB7 (β-catenin) antibody diluted 1:200, in PBS, at room temperature for 1 hour. Cells were washed 3 times in PBS. For immunofluorescent staining for cadherin -11 or E-cadherin, Fluorescein conjugated anti-mouse secondary antibody (Kirkegaard and Perry Laboratories) was applied at room temperature for 1 hour. Cells were washed 3 times with PBS. To visualize β-catenin, Texas Red Phalloidin secondary antibody (Kirkegaard and Perry Laboratories) was used. Cells were

washed 3 times in PBS and subsequently mounted on microscope slides using Vectashield mounting medium. Microscopy was completed on a Nikon 3600 fluorescent microscope.

***In vitro* cell invasion assay:** Standard 48-well Boyden chamber assays were prepared by placing DMEM supplemented with 15% foetal bovine serum into the bottom wells of the chamber as a chemoattractant. After coating 8μm pore-size polycarbonate filter with 20 μg/ml matrigel, $1x10^4$ serum-starved cells in serum-free DMEM with or without peptide treatment were placed in the upper chamber and incubated at 37°C for 16 hours. Membranes were removed and cells fixed using the DiffQuik staining kit for 5 minutes, followed by cytoplasmic staining for 3 minutes and nuclear staining for 5 minutes. Cells on the upper surface of the membrane were swiped with a cotton swab. Membrane were air-dried overnight before being dessicated in 100% xylenes for 90 minutes. The membrane was mounted on a microscopy slide using Cyosol-60 and analyzed. The analysis included taking pictures of each cell invasion field using an Olympus Vanox microscope at x2.5 magnification and an eye reticle to locate the center of each field. Quantitation of cell invasion involved counting the number of cells in each picture taken using the Metamorph computer program. Each treatment was performed in triplicate and graphs represent the average number of cells counted including the respective standard deviation.

Real-time PCR: Cells were plated on 6 cm dishes at three densities $2x10^5$ (low density), $4x10^5$ (medium density), and $2x10^6$ (high density) and grown in 0.2% serum containing medium overnight. The following day, the cells are collected and RNA isolated using standard protocols.

The RNA collected is used to perform quantitative PCR (qPCR) experiments using primers designed by and purchased from Applied Biosystems Incorporated

RT-PCR analysis: Total cellular RNA from the MDA-MB-231 human breast cancer cell line was extracted with a TRIzol-based extraction method. One microgram of total RNA was reverse transcribed and amplified according to the protocol supplied with the One-Step RT-PCR kit (Qiagen). The primers used are shown in **table 2** (Fitzpatrick *et al.*, 2003; Kurebayashi *et al.*, 1999).

Transcript	Forward sequence	Reverse sequence	TM (°C)	Number of cycles	Product length (bp)
VEGF-A	5'-GCA GAA TCA TCA CGA AGT GG-3'	5'- GCA TGG TGA TGT TGG ACT CC-3'	58	35	212
VEGF-D	5'- GTA TGG ACT CTC GCT CAG CAT-3'	5'- AGG CTC TCT TCA TTG CAA CAG-3'	57	35	261

Table 2: Primer sequenced of VEGF-A and VEGF-D used in RT-PCR experiments.

VEGF-A or VEGF-D ELISA immunoassay: Cells were plated at increasing cell concentrations in 1ml serum-containing DMEM in 12-well plates. The cell culture-supernate was collected after 24, 48 and 72 hours and the cells were counted. Cell culture supernates were analyzed according to the protocol for the Quantikine human VEGF or human VEGF-D ELISA kit (R&D Systems). In brief, cell culture supernates were incubated in pre-prepared antibody coated 96 -well plates for 2 hours. Following 3 washes using the appropriate wash buffer, wells were further incubated with a second conjugate antibody as described for the classic sandwich ELISA method. After 3 more washes the amount of protein detected was visualized and the $O.D._{450\text{-}540}$ measured on a fluorimeter to obtain the relevant protein content for each sample using a standard curve.

Cadherin-11 regulation by cell density, β-catenin and TGFβ:
MDA-MB-231 cells were plated on 6 cm dishes at three densities $2x10^5$ (low density), $4x10^5$ (medium density), and $2x10^6$ (high density) and grown in 0.2% serum containing medium overnight. The following day, the cells are collected and RNA isolated using standard protocols.

MDA-MB-231 cells were plated on 6 cm dishes at a density of $2x10^6$ cells per plate (high density). Further, cells were grown in 0.2% serum containing medium overnight. On the next day cells were pretreated with 10uM Alk5 inhibitor (SB431542) or DMSO for 30 minutes prior to treatment. Cells were subsequently treated with 20mM LiCl or 20mM NaCl, and/or 5ng/mL TGFβ1. Cells were collected and RNA isolated using standard protocols.

The RNA collected is used to perform quantitative PCR (qPCR) experiments using primers designed by and purchased from Applied Biosystems Incorporated.

Results

Cadherin-11 wild-type (WT) inhibition with RNAi: In order to evaluate the role of cadherin-11 and its isoform in the process of breast cancer cell invasion, experiments were designed to analyze the effects of cadherin-11 inhibition on cell invasion. As part of this aim, small inteference RNAs were designed which were subsequently tested for their specificity and effect in inhibiting cadherin-11 WT and its variant form. Western blot analysis of MDA-MB-231 whole cell extracts showed a significant decrease in endogenous cadherin-11 protein expression following transient transfection of siRNA into cells (**Fig. 1**). The inhibiting effect of the siRNA was found to persist for 5-7 days.

A decrease in cadherin-11 WT expression was also observed after treatment with siRNA that inhibited both, cadherin-11 and its variant. However, 5B2H5 specifically recognizes cadherin-11 WT but not the variant form. Hence, any decrease in expression must be attributed to the inhibition of cadherin-11 only. No decrease in expression levels of cadherin-11 WT, compared to the mock-transfected cells was observed using siRNA targeted against the variant form.

Down-regulation of cadherin-11 following stable transfection with a cad-11 specific RNAi vector construct: MDA-MB-231 cells were stably transfected with the cadherin-11 WT specific RNAi vector construct. Following selection in hygromycin B containing medium, a number of clones was pooled to provide the subsequent pooled stable cell lines (1A - 10A). Western analysis was performed to investigate the extent of cadherin-11 WT down-regulation and three pooled stable cell lines were selected for further experiments, exhibiting levels of cadherin-11 WT down-regulation of 50% (4A) to close to 100% (6A) (**Fig. 2**). In addition to cadherin-11 WT RNAi pooled stables, empty vector (1B-4B) as well as hygromycin vector alone (1C), controls were produced. The resulting cell lines did not exhibit any degree of cadherin-11 WT down-regulation. Further, HEK 293 cells, transiently transfected with cadherin-11 WT and cadherin-11 VAR plasmid, were used as a positive control to ensure the specificity of the cadherin-11 WT detecting antibody (**Fig. 2**)

Down-regulation of cadherin-11 results in changes in cell morphology: Following the establishment of cadherin-11 WT down-regulating pooled stable cell lines, we were interested in examining the effects of cadherin-11 WT down-regulation on cell morphology. It was found that cadherin-11 WT down-regulation results in a more round cell morphology together with the appearance of cellular extensions. It has been suggested that both morphological changes might occur as a result of loss of cell - ECM adhesion (**Fig. 3**).

β-catenin remains localized at cell membrane despite of loss of cadherin-11 WT expression: An alternative method was employed to verify the down-regulation of cadherin-11 protein expression as well as analyze the effects on localization of cadherin-11 WT and β-catenin as a representative of cadherin-11 binding proteins. A change in β-catenin localization was expected as previous reports have shown that upon loss of E-cadherin, β-catenin pools diffuse away from the cell membrane into the cytoplasm. In view of the fact that MDA-MB-231 cells contain solely cadherin-11 and cadherin-11 VAR and no other cadherins, loss of cadherin-11 WT would be expected to result in a similar phenomenon. Subsequent immunocytochemistry double-staining confirmed the down-regulation of cadherin-11 and indicated a shift from primarily cell membranous localization to predominantly nuclear or localization within the Golgi bodies (**Fig. 4**).

With respect to β-catenin it was found that β-catenin localization persists at the cell membrane, leading to the question what alternative cell membrane protein binds β-catenin (**Fig. 4**). It was suggested that E-cadherin might have become up-regulated following the down-regulation hence binding β-catenin to the cell membrane.

No up-regulation of E-cadherin in cadherin-11 WT specific RNAi pooled stable cell lines: In view of the results obtained within the above experiment, the pooled stable cell lines were double-stained for E-cadherin and β-catenin to investigate any potential up-regulation of E-cadherin in relation to cell membrane localization of β-catenin. However, results showed that E-cadherin is not up-regulated in the pooled stable cell lines examined, and that β-catenin remains localized to the cell membrane (**Fig. 5**).

Following these interesting results, it was suggested that β-catenin might be able to bind protein components of tight junctions such as occludin or claudins. As an alternative suggestion, it was considered that β-catenin binds to the actin-cytoskeleton via α-catenin.

Analysis of invasive potential of cad-11 specific RNAi pooled stable cell lines: In addition to establishing a link between cadherin-11 WT down-regulation and downstream events, it was of interest to examine the effect of cadherin-11 down-regulation on the invasive potential of breast cancer cells. To this end cell invasion assays based on the Boyden method were performed. The results did not reveal a significant increase of invasive capacity within cells that have cadherin-11 down-regulated. However, a trend became apparent with experimental repeats, indicating an increase in invasive potential as a result of cadherin-11 WT down-regulation (**Fig. 6**). Nevertheless, this trend needs to be verified through further experiments. In addition, significant inter-experimental variation, as illustrated by the very different scales on which the number of invaded cells was measured between the repeat experiments, needs to be eliminated. This will be achieved by plating the cell out at equal densities and serum-starvation overnight, before the start of the cell invasion experiment.

Cadherin-11 WT and cadherin-11 variant antibodies: Previously, efforts to analyze cadherin-11 WT and cadherin-11 variant expression have been hampered due to a number of factors including low protein expression levels, limited antibody specificity and limitations in the use of working antibodies in a variety of assays. Hence, a primary aim of the project was to identify working antibodies against cadherin-11 WT and its splice variant form for use in immunoblotting, immunocytochemistry and immunohistochemistry. Aspects of this work were successfully completed previously. For example Feltes *et al.* demonstrated the use of the ICOS 113H and 5B2H5 antibody for immunoblot and immunocytochemistry assays (Feltes *et al.*, 2002). However, no previous reports show the use of an antibody specific for the cadherin-11 splice variant. Here, we show immunoblot analysis of cadherin-11 variant expression using a specific antibody that recognizes the truncated intra-cellular domain of the splice variant. HEK 293 (human embryonic kidney) cells were transiently transfected with a cadherin-11 variant expressing plasmid. These cells lack

endogenous cadherin-11 WT and cadherin-11 variant and therefore provide a good system for testing a number of anti-cadherin-11 variant antibodies. Cells were harvested after 72 hours and Western blot analysis showed a cadherin-11 variant specific doublet band (**Fig. 7**). Further investigation into the specificity of the cad-11 variant antibody's ability to detect endogenously expressed cad-11 variant yielded no clear positive result as yet (**Fig. 7**). However, a protein band specific to the breast cancer cell lines Hs578T and BT549 was detected and although it was significantly smaller in size than the exogenously expressed cadherin-11 variant protein, further analysis is required to either confirm it being specific to endogenous cadherin-11 variant or definitively exclude the possibility of the band being a breakdown product or an additional splice variant of cadherin-11 (**Fig. 7**).

Preliminary experimental result analyzing cad-11 VAR mRNA expression with respect to cell density: As previous experimental attempts to examine protein expression levels of cadherin-11 VAR using a specific antibody have failed, the alternative method of real-time PCR was employed. Within a preliminary experiment, real-time PCR was used to measure the relative expression levels of cadherin-11 VAR mRNA. Cadherin-11 VAR specific PCR primers were designed to detect only cadherin-11 VAR mRNA. The result shows cadherin-11 VAR mRNA expression to increase in relation to an increase in cell density. This result confirms previous observations made by Feltes *et al.*, 2002 (**Fig. 8**).

Cadherin-11 inhibition with small synthetic peptides: The amino acid sequence His-Ala-Val (HAV) located in the C-terminal of the extracellular domain was implicated in the interaction between cadherins as synthetic peptides containing the sequence effectively blocked mouse embryo blastomere assembly (Blashuk *et al.*, 1990). However, the HAV sequence was found to be only present in type I classical cadherins such as N-, P- and E-cadherin. Instead, cadherin-11 as a type II classical cadherin contains the sequence QAV (Blashuk *et al.*, 1990). Attempts were made to design small synthetic peptides specific for cadherin-11. Following the design, based on stability and solubility, the most promising peptides were selected and tested for their inhibitory ability in standard *in vitro* cell invasion assays.

As shown in **Figure 9**, ADH93 inhibited MDA-MB-231 cell invasion through a matrigel- coated membrane at peptide concentrations of ≥0.6mg/ml. In contrast, ADH92 did not have an observed effect on cell invasion. The effect of ADH114 and ADH113 are shown in **Figure 9**. A marked decrease in the number of cells invading the matrigel-coated membrane was observed using ADH114. An approximately 50% reduction in cell invasion occurred at 0.6mg/ml peptide concentration and was maintained at higher concentrations. On the contrary, ADH113 did not result in a significant decrease in cell invasion. The range of peptide concentrations tested revealed ADH93 and ADH114 to inhibit 50% of cell invasion at ~0.6mg/ml peptide concentration. Concentrations above 1mg/ml were not analyzed, as the majority of peptides were not soluble at higher concentrations (**table 3**).

Name	50% inhibition
ADH92	>1mg/ml
ADH93	~ 0.6mg/ml
ADH114	~0.6mg/ml
ADH113	> 1mg/ml
ADH549	~0.6mg/ml
ADH548	>1mg/ml
ADH243	not supplied

Table 3: Summary of cadherin-11 inhibitory peptides. Level of activity is expressed as peptide concentration resulting in approximately 50% inhibition of cell invasion.

Analysis of ADH549 and its inhibitory effect on MDA-MB-231 cell invasion, gave a similar result to ADH93 and ADH114. Again, an approximately 50% reduction in cell invasion was observed at ≥0.6mg/ml peptide concentration (**Fig. 10**). To support the experimental result, pictures of the cell invasion spots on the membrane were taken. These clearly show the marked reduction in cell invasion between 0.4 and 0.8mg/ml peptide concentration. However, to verify the specificity to cadherin-11 of the peptides, all peptides were tested on the MDA-MB-435 cancer cell line that was found to only express N-cadherin (**Fig. 10**). The results showed that ADH549 is not specific for cadherin-11 as it also inhibits cell invasion in the MDA-MB-435 cells and hence was suggested to inhibit cadherin-11 as well as N-cadherin. In conclusion, three peptides, namely ADH93, ADH114 and ADH549 were shown to cause a decrease in cell invasion of MDA-MB-231 cells at ≥0.6mg/ml concentrations. In contrast, ADH92, ADH113 and ADH548 did not reveal any effect on cell invasion.

VEGF-A and VEGF-D expression in breast cancer cells: In order to confirm previous reports of VEGF-A and VEGF-D expression in MDA-MB-231 breast cancer cells, semi-quantitative RT-PCR was carried out on total RNA (Kurebayashi *et al.*, 1999). As shown in **Figure 11** both, VEGF-A and VEGF-D template mRNA were detected in MDA-MB-231 cells demonstrating that VEGF-A and VEGF-D are expressed. nUsing ELISA immunoassays the expression of secreted VEGF-A and VEGF-D in MDA-MB-231 cells was examined. Results showed a cell density-dependent increase in VEGF-A expression after 24 hours of cell growth. Similar results were obtained for samples analyzed after 48 and 72 hours of growth.

No results were obtained for expression levels of VEGF-D, as levels of secreted VEGF-D protein appear not high enough for detection by the ELISA kit. Further, analysis of MDA-MB-231 cell lysates to detect protein levels of non-secreted VEGF-D did not yield any result either.

Decrease in VEGF-A expression following peptide treatment: Using the VEGF-A specific ELISA immunoassay, VEGF-A protein expression was analysed following treatment with the cadherin-11 specific inhibitory peptide, ADH93 and its respective control peptide ADH92. The results show that VEGF-A was decreased after treatment with either peptide. However, the decrease was more pronounced with ADH93 (**Fig. 12**). Further experiments are required to analyze VEGF-A expression levels with respect to the other cadherin-11 specific inhibitory peptide.

Cadherin-11 regulation by cell density, β-catenin and TGFβ:

Density. To determine if cadherin-11 expression is density dependent, MDA-MB-231 cells were grown at various densities (low, medium, high) for 16 hours in 0.2% serum containing medium. Quantative real-time PCR was used to assay cadherin-11 expression. Cadherin-11 expression in cells grown at high density was increased relative to cells grown at low density (Figure 13) as consistent with published data (Feltes et al., 2001).

TGFβ1. TGFβ family members are possible upstream components of the regulatory pathway involved in the transcription of cadherin-11. Previous work has demonstrated TGFβ1 negatively regulates cadherin-11 expression. In order to further investigate the role of TGFβ1 in cadherin-11 regulation, MDA-MB-231 cells were treated with TGFβ1 alone or in combination with a TGFβ type 1 receptor (Alk 5) inhibitor (SB431542). TGFβ1 treatment resulted in attenuated cadherin-11 expression, which was reversed when the cells were treated with the Alk5 inhibitor alone or in combination with TGFβ1 (Figure 14).

LiCl. Previous data had suggested that β-catenin down regulates cadherin-11 expression in a similar manner as TGFβ1. LiCl, a known inhibitor of GSK3β, prevents β-catenin phosphorylation and subsequent degradation. Once β-catenin is stabilized in the cell, it is able to act as a transcription factor of

various genes commonly activated during cancer development and progression. MDA-MB-231 cells treated with LiCl exhibited a drastic decrease in cadherin-11 expression (Figure 14). Additionally, previous data had suggested a secreted ligand was responsible for cadherin-11 down regulation, possibly TGF-β1. Therefore the cells were treated with the Alk5 inhibitor in addition to LiCl. When treated with this inhibitor the effect of the LiCl was unable to be reversed (Figure 14) suggesting that LiCl does not stimulate the cells to secrete additional TGFβ1 ligand.

Discussion:

Preliminary experiments have provided a number of important results on which to base further experimental progress. As the above experiments represent the preliminary stage of the investigation, additional experiments are required to further evaluate the effects of cell density and cadherin-11 inhibiting peptide exposure on VEGF-A expressing breast cancer cells. So far, results highlighted a distinct correlation between cell density and VEGF-A secretion in MDA-MB-231 cells. In addition, the potential influence of cadherin-11 and cadherin-11 expression on VEGF-A secretion resulted in interesting observations emphasizing the possible effects of cadherin-11 and cadherin-11 variant expression in conjunction with cell density on VEGF-A secretion.

However, future experimental design will need to address the problems related to the use of different cell lines and the potential effects resulting from different genetic backgrounds when analyzing the interrelation between expression and secretion of different proteins. In addition we have now established a real-time PCR assay to detect VEGF-D expression and will test the effects of the various cadherin-11 manipulations on VEGF-D mRNA levels. Our ultimate goal will be to analyze all available peptides for their effects on VEGF-A secretion in MDA-MB-231 cells and potentially establish a link between the peptides potential to inhibit cadherin-11 activity, to modulate VEGF-A secretion and the capacity for cell invasion.

Clearly, agents that inhibit the function of cadherin-11 are likely to prove useful agents in the treatment of breast cancer. In addition the more knowledge we have about the factors and conditions that regulate cadherin-11 expression the more likely we are to predict situations which lead to inappropriate expression. In this regard our data point to a role for cell density, wnt/beta catenin and TGF beta signaling in the regulation of cadherin-11 expression. Of considerable interest is the demonstration that whereas increasing cell density increases cadherin-11 expression, TGF beta and wnt/catenin signaling activity repress it.

Key Research Accomplishments:

1. Demonstration that cadherin-11 blocking small molecules can regulate VEGF expression by cadherin-11-expressing cells.

2. Cells that express cadherin-11 siRNA make very low levels of cadherin-11 but normal levels of cadherin-11 variant and are more invasive than control cells.

3. Cadherin-11 expression is repressed by cell density, beta catenin signaling and by TGF-beta.

Reportable Outcomes:

Hampel, C, Blaschuk, O, Rowlands, T. and Byers, S. Small molecule antagonists of cadherin-11 function alter breast cancer cell invasion and VEFG expression. In preparation

Conclusions:

Our data show that a particular class of inhibitors designed to block the ability of cadherin-11 to interact with the extracellular matrix does indeed affect cell invasion and the ability of cells to express VEGFs. Other molecules designed to disrupt the cell-cell adhesive function of cadherin-11 did not affect cell invasion or VEGF expression. In other studies we have generated cell lines expressing siRNA directed at both cadherin-11 and cadherin-11 variant. These data strongly indicate that inhibition of the ability of cadherin-11 to interact with the ECM blocks cell invasion. The demonstration that small molecule inhibitors can effectively block this important function of cadherin-11 bodes well for the development of drugs that can inhibit the ability of cadherin-11 expressing cells to stimulate blood vessel growth and metastasize. In the final year of the grant we will use these inhibitors and cells which do and do not express cadherin-11 in animal studies.

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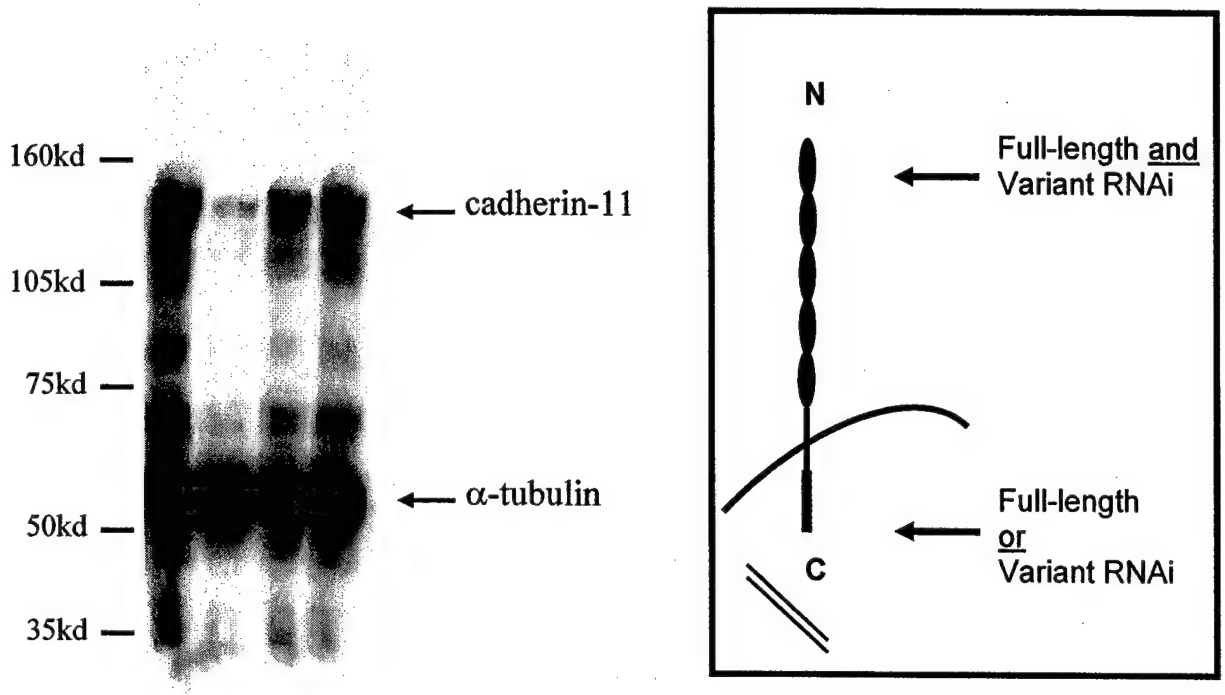

Fig. 1: Downregulation of cadherin-11 WT following 3 sequential transient transfections with cad-11 WT specific RNAi.
Western blot analysis was performed using a cad-11 WT specific Antibody to detect changes in protein level. 20μg of protein were Loaded in each well and α-tubulin was used as a loading control.

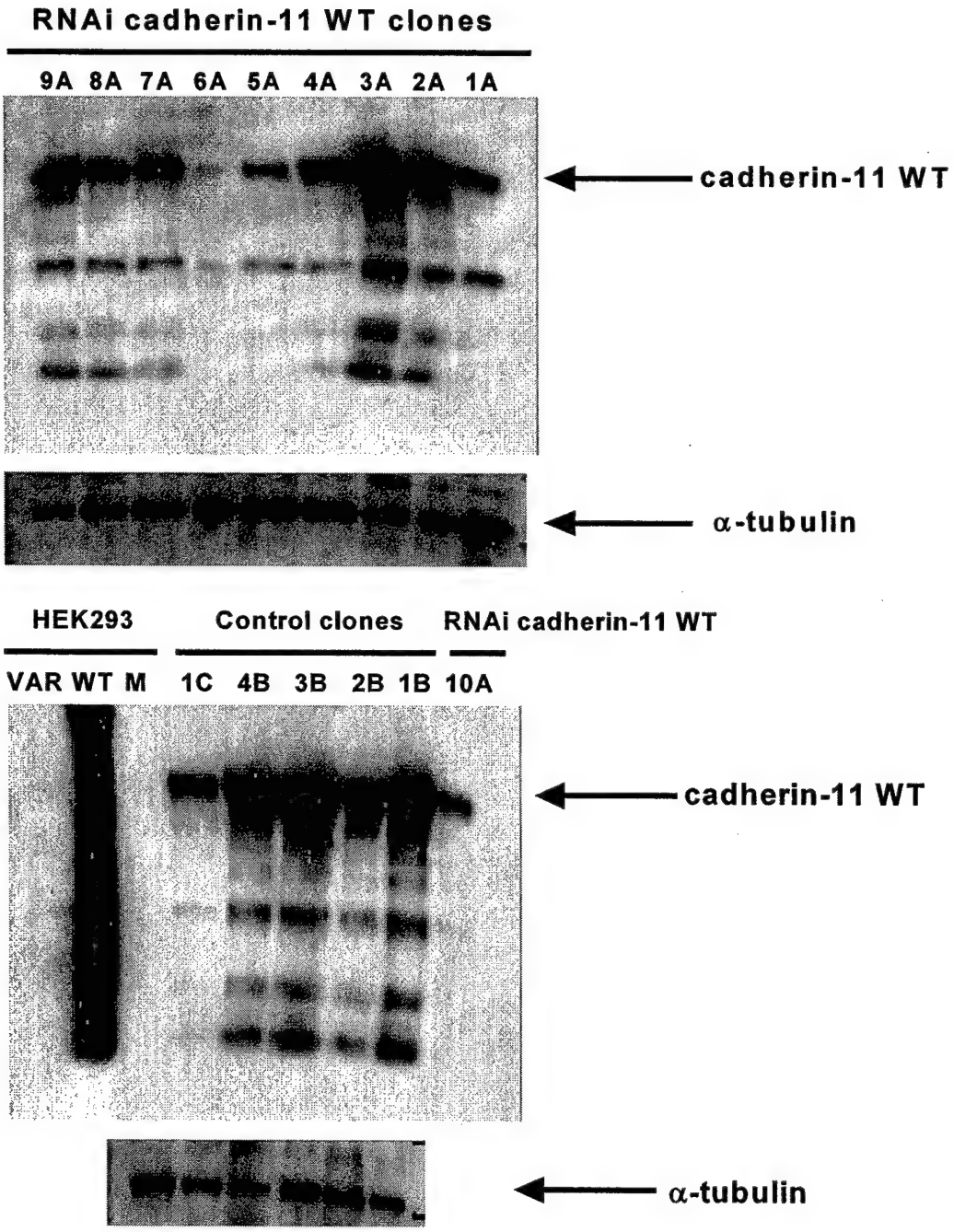

Fig. 2: Downregulation of cadherin-11 WT following stable transfections with cad-11 WT specific RNAi vector construct. Western blot analysis was performed using a cad-11 WT specific antibody to detect changes in protein level. 20μg of protein were loaded in each well and α-tubulin was used as a loading control.

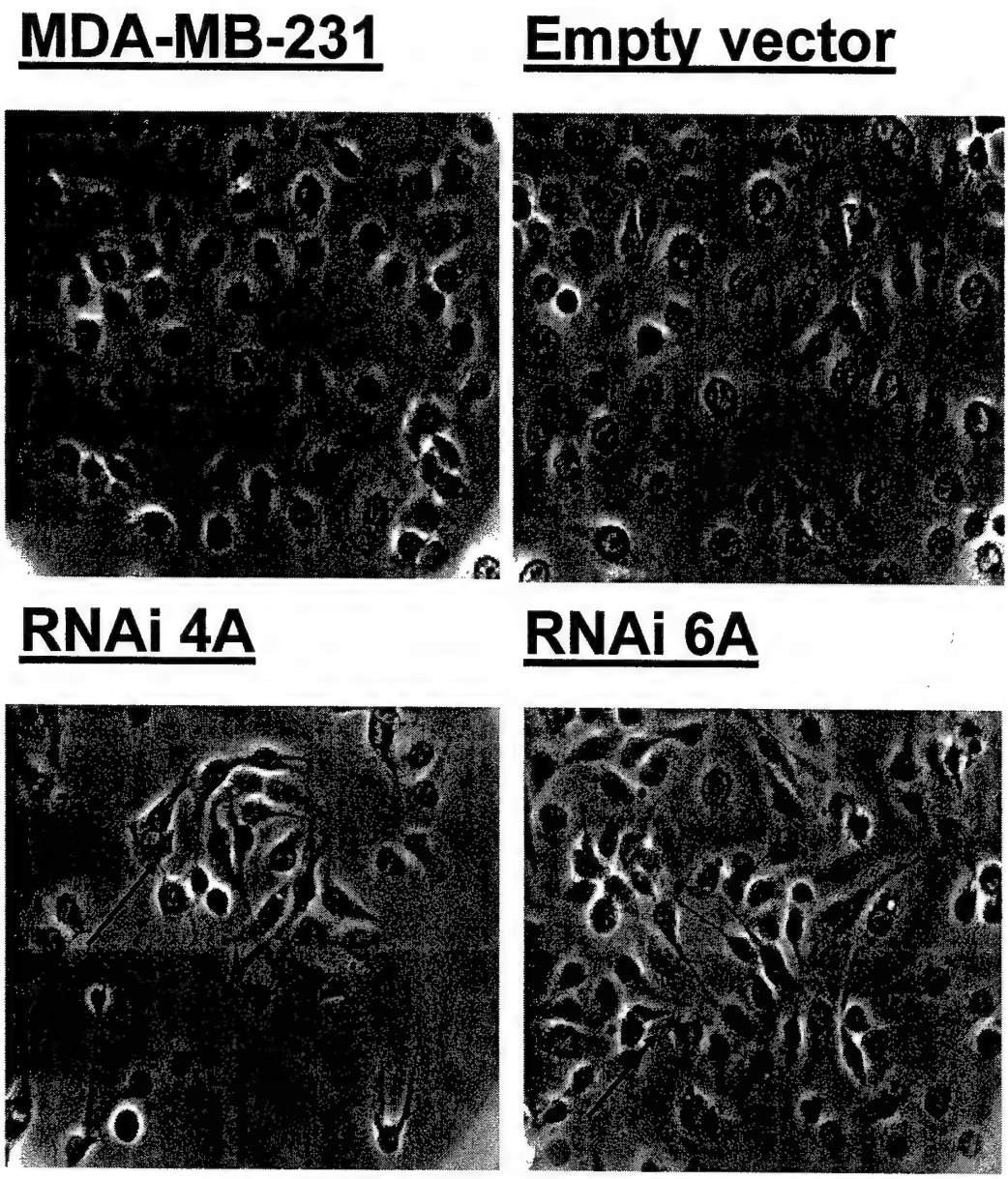

Fig. 3: Downregulation of cadherin-11 WT results in changes of cell morphology . Cells were plated on coverslips and phase contrast images (x20) taken.

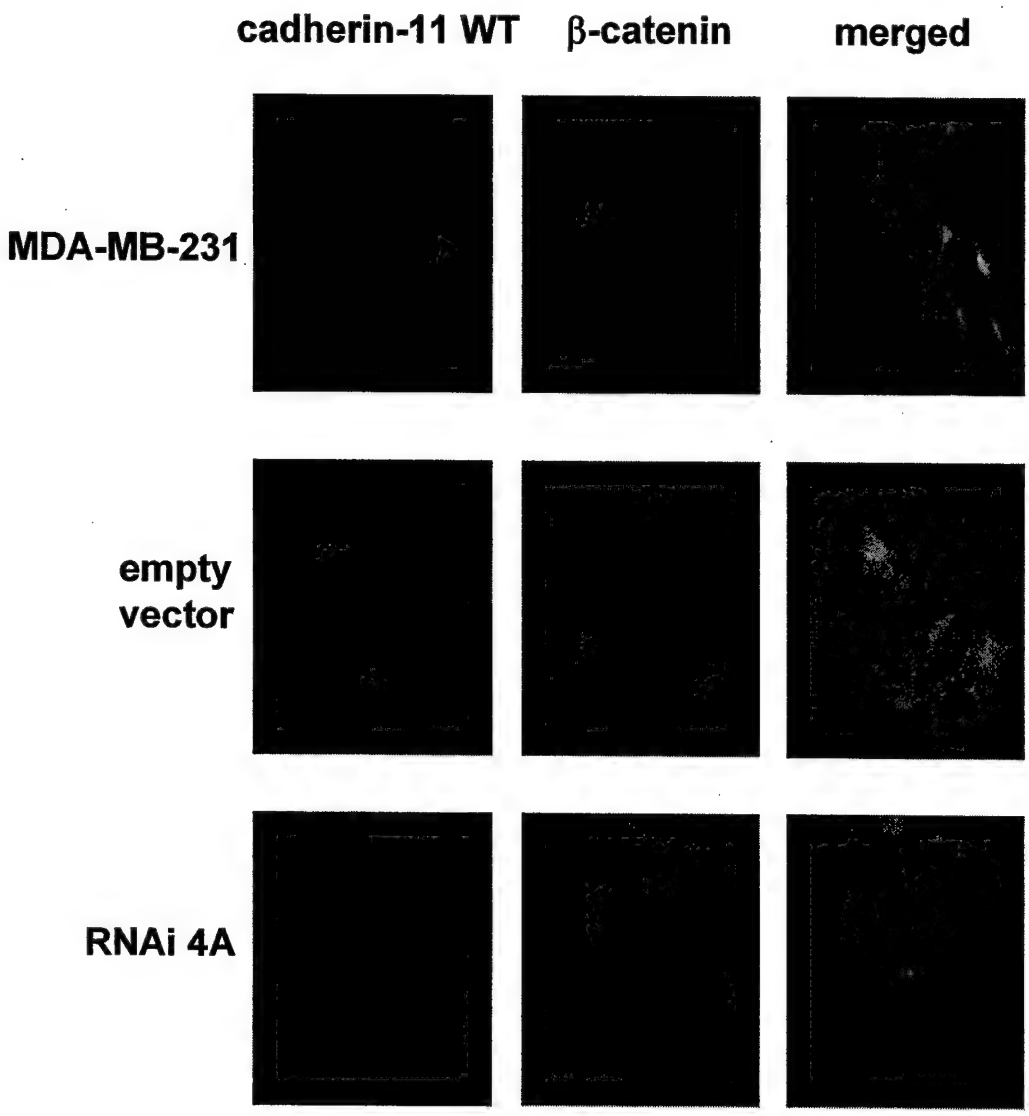

Fig. 4: Downregulation of cadherin-11 WT as shown by immunocytochemistry.
Cells were plated out on coverslips and subsequently stained with a cad-11 WT specific antibody and a β-catenin specific antibody.
β-catenin was found to remain localised at cell membrane although MDA-MB-231 cells do not known to express other cadherins except cad-11.

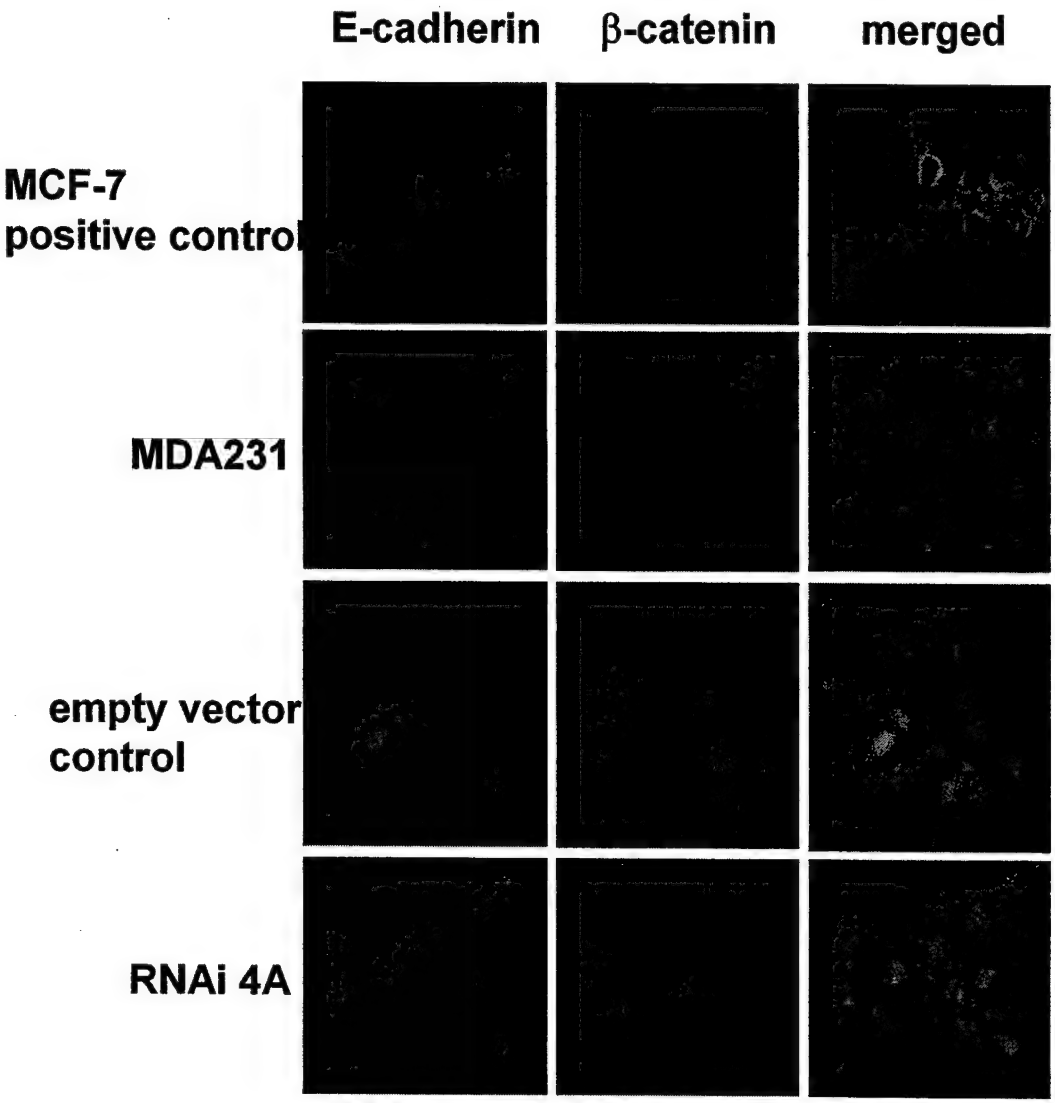

Fig. 5: No upregulation of E-cadherin as shown by immunocytochemistry. Cells were plated out on coverslips and subsequently stained with an E-cadherin specific antibody and a β-catenin specific antibody. β-catenin was found to remain localised at cell membrane despite no observed up-regulation of E-cadherin.

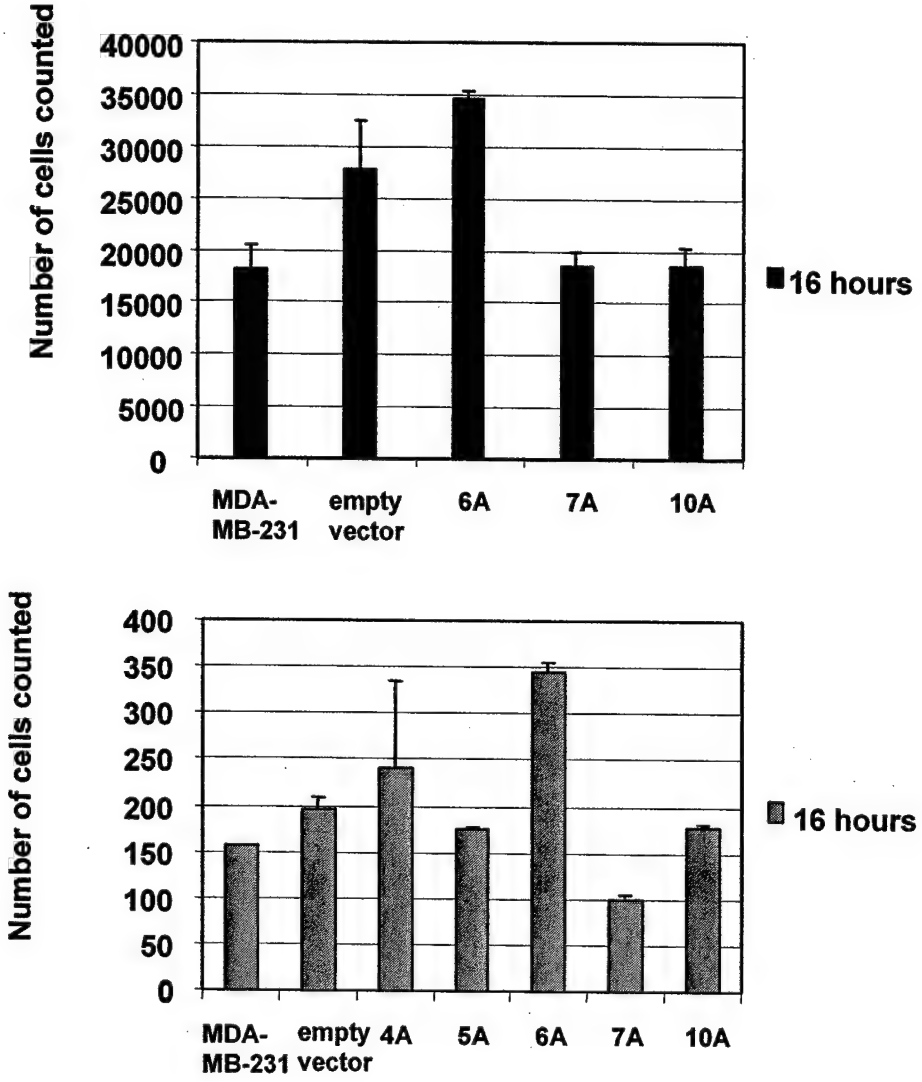

Fig. 6: Analysis of invasive capacity of cad-11 WT RNAi pooled stables as determined by using Boyden chamber cell invasion assay.
Cells were serum-starved overnight before being aliquoted at equal number into the Boyden chamber wells. Cells were allowed to invade the matrigel-coated membrane towards the chemoattractant for 16 hours.

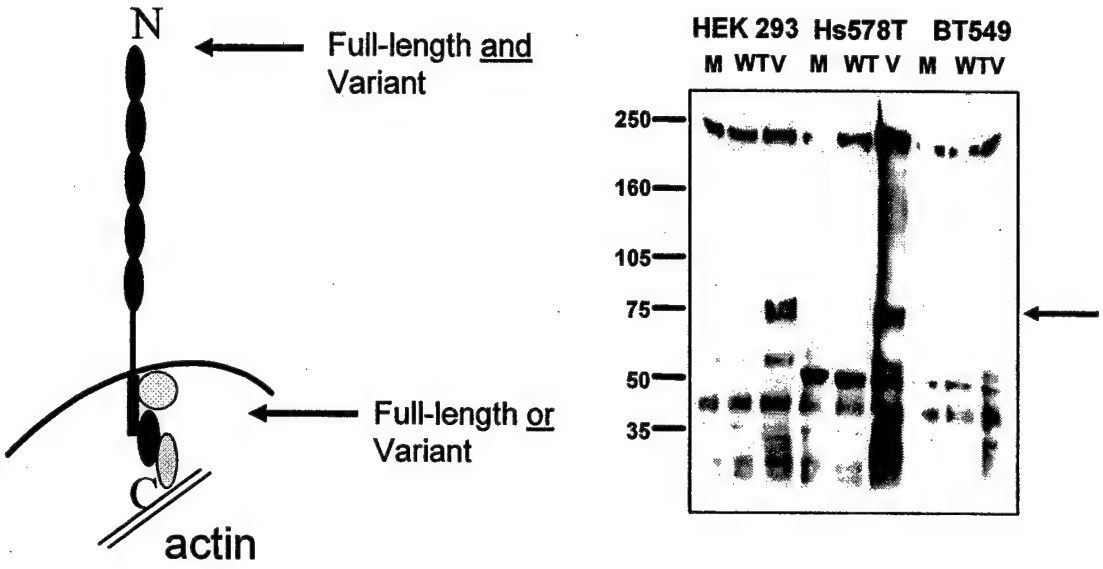

Fig. 7: Detection of exogenous but not endogenous cad-11 VAR protein using cad-11 VAR specific antibody.
Cells were transiently transfected with empty, cad-11 WT and cad-11 VAR vector Constructs to exogenously express the respective protein. Western analysis was performed. 20μg of total protein was loaded per well.

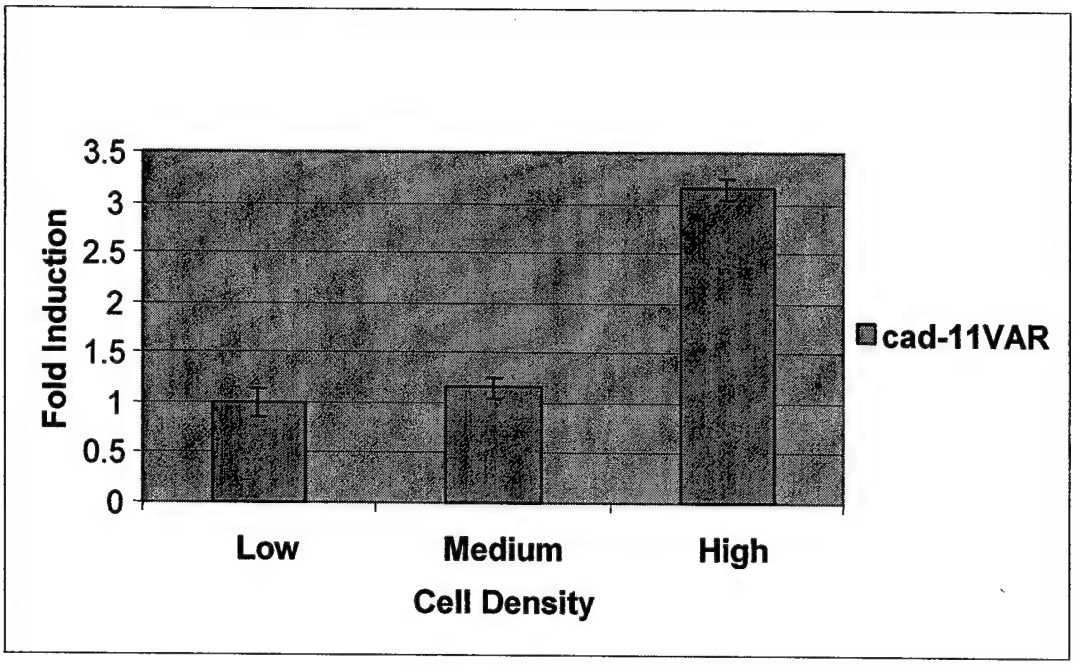

Fig. 8: Analysis of cad-11 VAR mRNA expression levels as determined by real-time PCR. mRNA was extracted from MDA-MB-231 grown at low, medium and high density before being analysed using custom-made PCR primers.

Cadherin-11 specific inhibitory peptide **Control peptide**

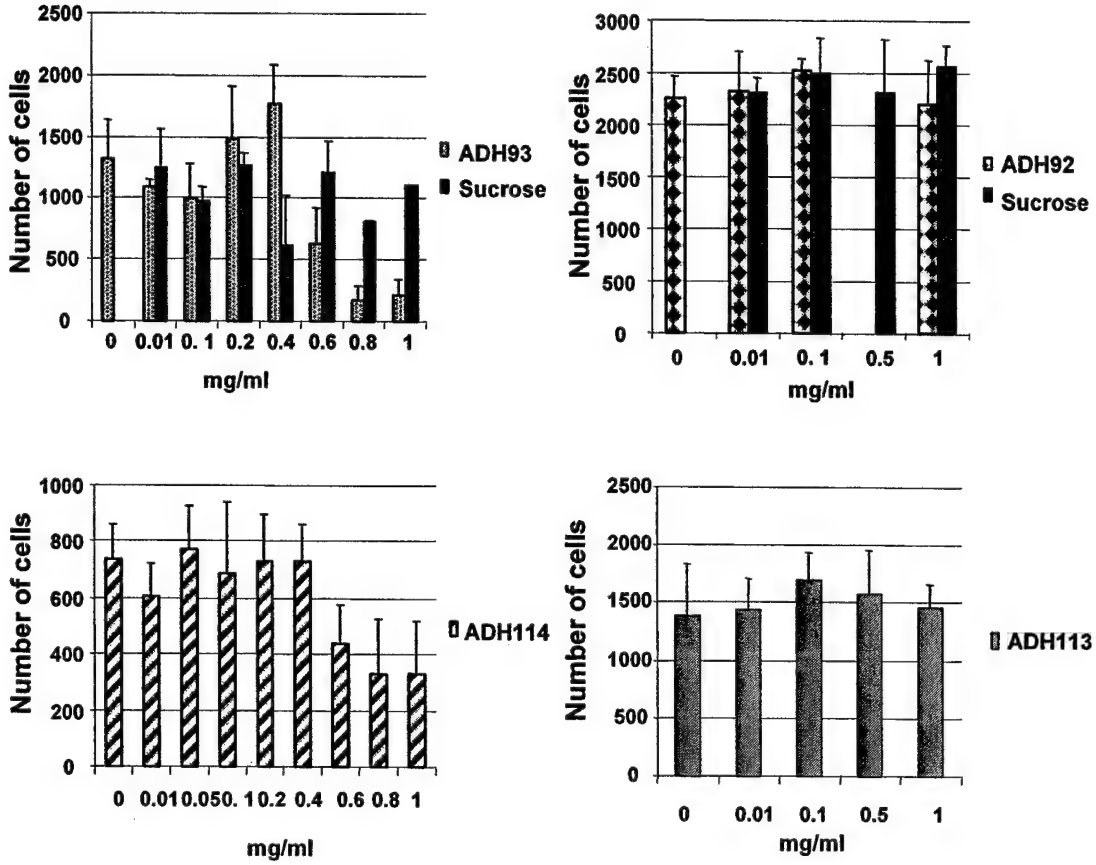

Fig. 9: Cadherin-11 specific inhibitory peptides result in inhibition of cell invasion with increasing concentrations. In contrast, the respective control peptides have no effect on the invasive potential of MDA-MB-231 cells.
Cells were serum-starved overnight before being dosed with peptide and allowed to invade through the matrigel-coated membrane within the Boyden chamber.

Inhibition of cell invasion			
MDA-MB-231		MDA-MB-435	
Name		Name	
ADH93	YES	ADH93	NO
ADH92 (CONTROL)	NO	ADH92	N/A
ADH114	YES	ADH114	NO
ADH113 (CONTROL)	NO	ADH113	NO
ADH549	YES	ADH549	YES
ADH548 (CONTROL)	NO	ADH548	N/A

Fig. 10:Summary of cad-11 inhibiting peptide results with respect to cell invasion. All peptides used are suggested to disrupt the cell-ECM interaction. In addition,ADH549 was found to be non-specific as it inhibits cell invasion in MDA-MB-435 cells (express only N-cad) and MDA-MB-231 (express only cad-11).

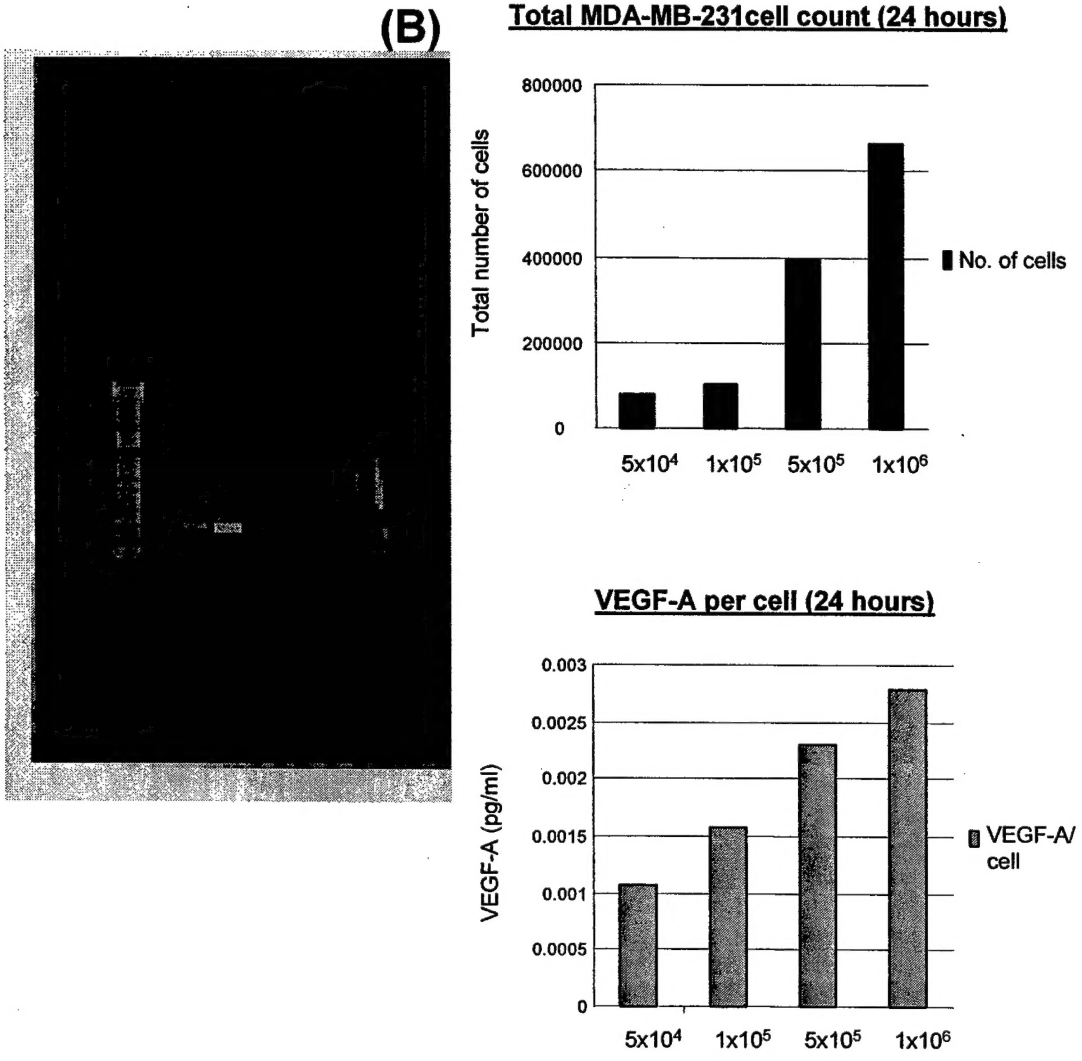

Fig. 11: VEGF-A increases with increasing cell density as determined by ELISA (B). Cells were plated out at increasing cell densities and the growth medium was harvested after 24 hours. The medium aliquots were analysed using a VEGF-A specific ELISA assay kit. Presence of VEGF-A in MDA-MB-231 cells was verified by RT-PCR (A).

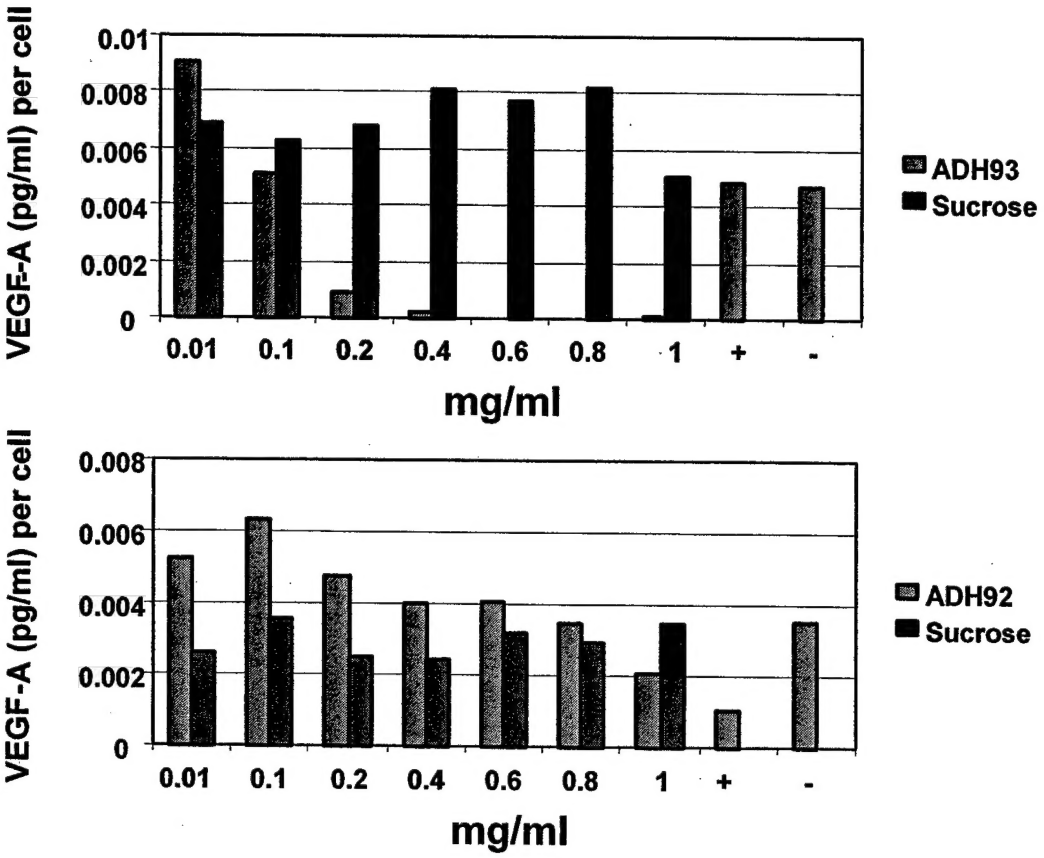

Fig. 12: VEGF-A expression decreases following treatment with cad-11 specific inhibitory peptides. Cells were plated out at equal cell density and treated with peptide for 16 hours. Subsequently, the growth medium was harvested and the medium aliquots analysed using a VEGF-A specific ELISA assay kit.

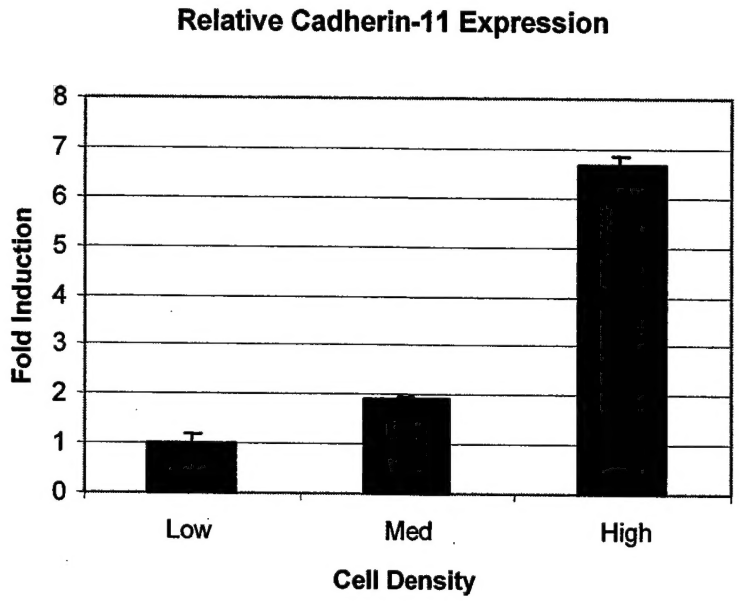

Figure 13. Cadherin-11 expression as a function of density. Cadherin-11 RNA expression was analyzed using quantitative real-time PCR. MDA-MB-231 cells were plated at three densities: low (40% confluent), medium (70% confluent), and high (95% confluent).

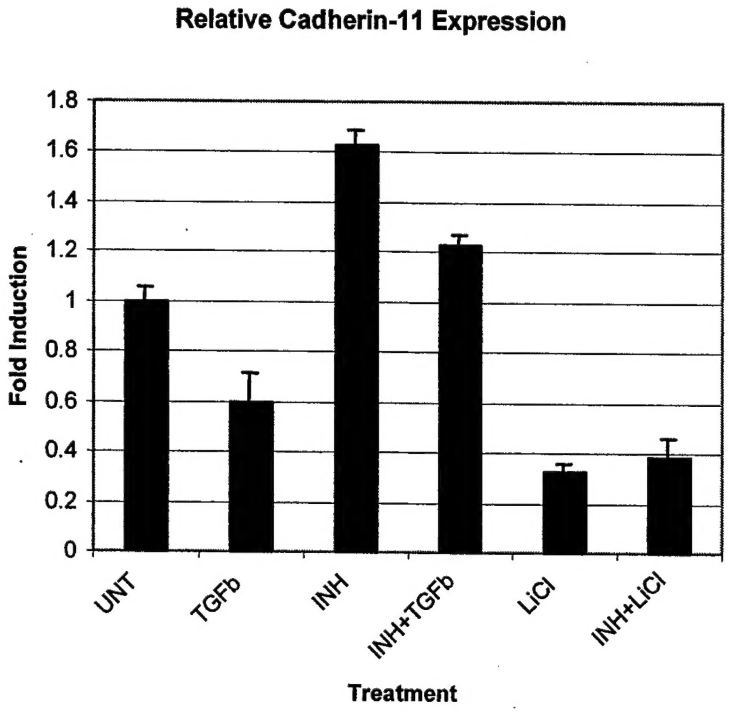

Figure 14. The effects of TGF beta and wnt/catenin signaling on Cadherin-11 expression. Cadherin-11 RNA expression was analyzed using quantitative real-time PCR. MDA-MB-231 cells were treated with 5ng/mL TGFb1 and/or 20mM LiCl (or 20mM NaCl in the non-LiCl treated cells).